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An effect of castration and testosterone replacement on a circadian pacemaker in mice *(Mus musculus)*

(circadian rhythm/activity)

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ABSTRACT **Castration of mice in freerunning conditions (total darkness, DD) causes a reduction of running wheel activity in the beginning of the active period (α) and stimulates activity at the end of α. Simultaneously, the period (τ) of the freerunning rhythm is increased. Both effects are abolished by implantation of a Silastic capsule from which a physiological dose of testosterone is released at a constant rate. The results are tentatively explained by differential endocrine influences on two oscillating components in the pacemaker of the circadian activity rhythm.**

Early work on endocrine involvement in circadian rhythms was mainly aimed at the localization of the clock within the various endocrine glands or within a cycle of nervous–endocrine–metabolic events (1). Much of the research was done in animals exposed to daily light-dark cycles, which precludes evidence on the endogenous nature of the rhythmic phenomena observed. From a series of experiments on blinded rats, Richter (2) stated that *freerunning* circadian activity rhythms were not impaired by any of the following interferences with the endocrine system: gonadectomy, adrenalectomy, hypophysectomy, hyper- or hypothyroidism, and pinealectomy. Apparently, the circadian clock driving the activity–rest cycle was not located in any of the glands involved. The demonstration by Andrews (3) that rat adrenals cultured *in vitro* continue to produce corticosteroids in a circadian rhythmic fashion considerably modified existing views in the field concerning the regulating function of a localized clock (4). Evidently, since at least one organ, and probably more, is capable of self-sustained oscillations, a centralized "clock" or "pacemaker" would serve to synchronize a number of peripheral oscillators rather than impose its rhythm on them. For instance, a circadian pacemaker apparently drives the rhythm of hypothalamic corticotropin-releasing factor (CRF) content (5), which presumably leads to a daily periodicity in adrenocorticotropic hormone (ACTH) production by the anterior pituitary, and the self-sustained adrenal rhythm is entrained by the oscillating plasma ACTH concentration (3). Richter (2) suggested that the circadian clock would be located somewhere in the hypothalamus of the brain. There is now evidence (6) pointing to the localization of the circadian pacemaker in the *nucleus suprachiasmaticus* of the anterior hypothalamus. This nucleus is presently the most likely candidate, in that it is the only structure in the hypothalamus receiving direct retinal projections (7) which presumably convey the stimuli entraining the pacemaker to the outside world.

With the emergence of these new views concerning the mammalian circadian clock, the question of endocrine involvement no longer concerns the issue of clock localization, but whether there is any feedback regulation from peripheral organs that might be involved in its homeostatic properties (8). Is the high degree of stability and precision of freerunning circadian rhythms partly the result of coupling and feedback between an array of central and peripheral oscillators, or is it exclusively an intrinsic feature of the central pacemaker itself?

Recent experiments by Gwinner (9) show that pharmacological doses of testosterone can cause a severe disruption ("splitting") of the circadian activity rhythm in starlings. This led us to reinvestigate the effects of gonadectomy and subsequent testosterone replacement on a mammalian circadian rhythm, as a first approach to the questions of peripheral feedback.

MATERIALS AND METHODS

Twelve mice (*Mus musculus* C57Bl/6S), born March 12–14, 1974, were obtained from Simonson Laboratories (Gilroy, Calif.) and housed individually in wire mesh cages (23 × 19 × 33 cm), each in a ventilated light-tight box. The cages were equipped with running wheels, and an Esterline-Angus event recorder was used to monitor wheel revolutions. The animals were kept in constant darkness (DD) for the duration of the experiment (May 13, 1974–February 8, 1975). Feeding (Berkeley Diet, and tap water ad lib.) and cleaning were done once a week in low intensity red light (>610 nm), which does not interfere with the circadian rhythm. White light interruptions (2 hr) for all animals occurred on three occasions: May 28, when six mice were castrated and six received a sham operation; October 4, when the sham group was castrated; and December 11, when six animals (three from each group) received a subcutaneous implant of a polydimethylsiloxane† capsule (outside diameter 3.175 mm, inside diameter 1.575 mm; length 6 mm) containing crystalline testosterone, while the other six received a similar but empty capsule (10). Both testosterone and empty capsules had been incubated for 48 hr in a shaker water bath at 38°. The incubation medium (phosphate-buffered saline, pH 7.4) was changed three times a day and the capsules were implanted immediately after the last change. All operations were performed with sodium pentobarbital anaesthesia (85 mg/kg, intraperitoneally) and followed by an intramuscular penicillin injection.

Thus, in each of four groups, three animals were exposed to the same protocol. The complete activity record of one animal from each group is shown in Fig. 1. Except for one mouse that died following testosterone implantation, the ani-

Abbreviations: L and D, periods of light and darkness.

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† Silastic®, Dow Corning.

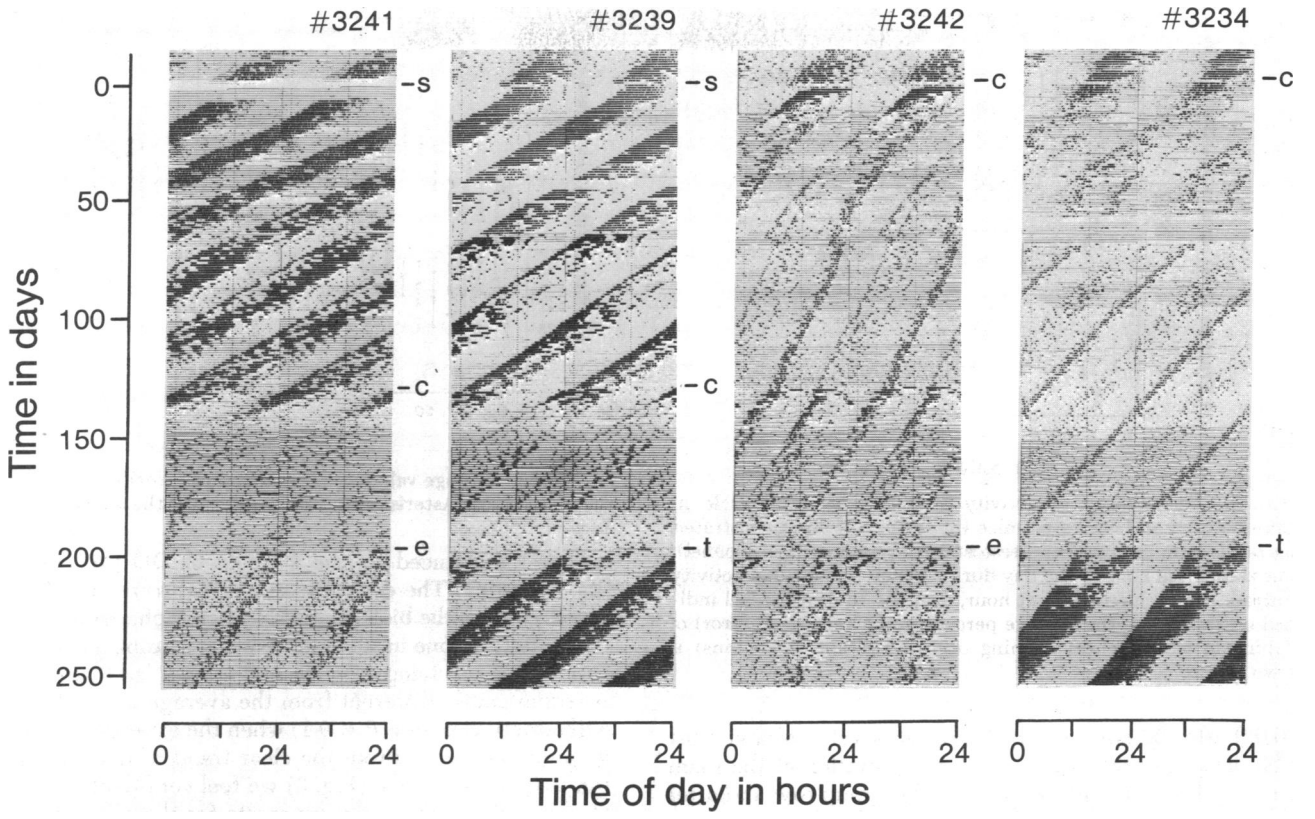

FIG. 1. Complete activity records of four mice, each representative for a group of three. The records are double plotted in the standard way. S = sham operation; C = castration; e = implantation of empty Silastic tubes; t = implantation of Silastic tube containing testosterone.

mals survived in good health. The experiment was terminated prematurely on February 8, 1975 by a fire which damaged the recorders. At autopsy seminal vesicles (without coagulating glands) and ventral prostates were removed and weighed. The results shown in Table 1 (data from intact males included for comparison) indicate that the testosterone implant (appropriate size determined from unpublished dose–response data) maintained a physiologically effective level of blood testosterone for the duration of the experiment.

The activity records were pasted day below day and double plotted in the standard way (Fig. 1). The circadian period (τ) was measured by eye-fitting a straight line through the onset of activity for each 10-day segment of the 255 days of the experiment. Ninety-five percent of such eyefits are less than 0.12 hr different from τ values calculated by linear regression (8).

RESULTS

The raw data (Fig. 1) show that the total amount of wheel running activity per cycle, as judged from the density of the records, is greatly reduced following castration. Implantation of testosterone almost immediately reestablished activity densities to levels characteristic of noncastrated animals. In addition, castration produces a qualitative change in the distribution of activity in each circadian cycle. Untreated and sham-operated mice, as well as castrated mice with a testosterone implant, had sharp onsets of activity, followed by a dense activity pattern, gradually fading out into the rest-time of the cycle. The pattern in castrated animals, both with and without an empty capsule, is characterized by an ill-defined onset of activity and a pronounced peak at the end of the active period. A quantitative analysis of two 10-day sections of the steady-state activity rhythms (Fig. 2) shows this in more detail. The activity time is clearly increased by castration and shortened by testosterone treatment. The activity peak which occurs 13–15 hr after the onset of activity in castrated animals is not seen in either sham-castrated or testosterone-implanted animals.

Fig. 1 suggests that in castrated mice the period (τ) of the circadian rhythm is shorter than in sham-operated mice. In the two animals (nos. 3239 and 3241) where a prolonged freerun preceded gonadectomy, τ gradually lengthened following the operation. Implantation of testosterone resulted in a decrease of τ (animals nos. 3234 and 3239). No response is seen in castrated mice which received an empty capsule (nos. 3241 and 3242). In several instances a phase shift of the rhythm is seen in association with any of the operations. This would be expected as an effect of the 2 hr of light all animals received on these occasions.

Fig. 3, based on all τ-values measured in 10-day intervals, shows that the operations on day 0 were followed by a decline in τ in the sham group and a rise in the castrated group. The difference between the averages, significant (P

Table 1. Effects of castration and testosterone capsule implantation on androgen-dependent sex accessory gland weight (Means ± SE)

Treatment	Number of animals	Seminal vesicle (mg)	Ventral prostate (mg)
Castration	6	5.7 ± 0.5	1.2 ± 0.2
Testosterone	5	45.5 ± 3.9	8.8 ± 1.2
Intact	4	42.1 ± 4.2	7.3 ± 1.4

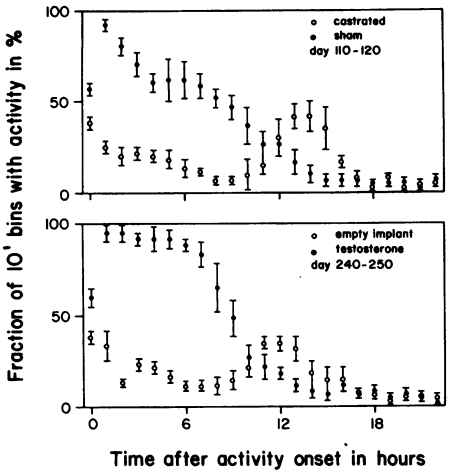

FIG. 2. Distribution of activity over the circadian cycle in sham-operated and castrated mice (upper panel) and in castrated mice with sham implants and testosterone implants (lower panel). Hour zero is the hour of the day during which the onset of activity occurred. For each subsequent hour, over the 10 day interval indicated on the figure, the average percentage (±1 standard error) of 10′ bins in which wheel running occurred (100% = 60 bins) is shown.

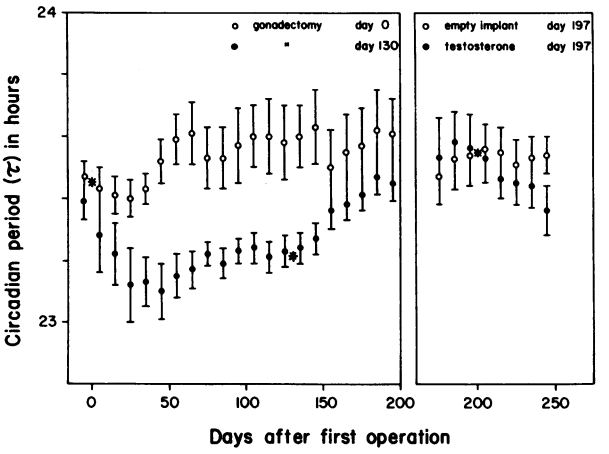

FIG. 3. Average value of τ (±1 standard error) in the course of the experiment. Asterisks indicate the dates of the operations.

< 0.02) after 30 days, reached a constant value of about 0.4 hr 60 days after the operation. After castration of the sham group on day 130, the average τ in this group rose and after 20 days became statistically indistinguishable from the other group. The 12 animals were then redivided and received the implants, which produced no significant change in the six animals where the capsules were empty, and a decrease in τ in those five where the capsules contained testosterone. The changes in τ calculated from 10-day periods before and after each of the operations are given in Table 2. Significant changes are associated with the sham operation on day 0 and castration on day 130. The decline in τ over the first month following sham operation on day 0 should not be interpreted as a response to the operation: this phenomenon is characteristic of intact animals. When released from an LD 12:12 (12 hr light:12 hr dark) cycle into DD, mice generally show initially a freerunning period closer to 24 hr than the steady-state τ they eventually assume in a prolonged DD-run (see, e.g., Fig. 19 in ref. 8). This "after-effect" apparently was insufficiently reduced by the 2 weeks of DD preceding the first operation. The discontinuation of this trend in the castrated group is the biologically significant phenomenon.

The testosterone implantation, while causing a decrease in τ in all five surviving animals, had not yet led to an average $\Delta\tau$ significantly different from the average $\Delta\tau$ in the group with empty capsules ($P < 0.1$) when the experiment came to its premature end. From the clear trend of decreasing τ in the testosterone group (Fig. 3) we feel confident that testosterone replacement can compensate for the effect of castration on τ.

DISCUSSION

Thus, removal of the testes in the house mouse (*Mus musculus*) has three effects on the circadian activity rhythm in DD: *(i)* a lengthening of the freerunning period, τ, *(ii)* a reduction of the total amount of activity per cycle [confirming earlier studies on rats in LD (11, 12)] *(iii)* a shift in the distribution of activity, producing a peak at the end of the activity time. For all practical purposes it is reasonable to assume that by castration we have taken away the influence of testosterone on the pacemaker governing the circadian activity rhythm. Subcutaneous implantation of capsules, which release physiological amounts of testosterone at fairly constant rates, restores the normal activity rhythm.

Table 2. Effects of castration and testosterone capsule implantation on the freerunning period (τ), in hr, of the circadian activity rhythm

Operation:	Date:	n	τ_1 before	τ_2 after	$\Delta\tau = \tau_2 - \tau_1$
Castration	May 28	6	23.47 ± 0.12	23.52 ± 0.17	0.06 ± 0.06
Sham	May 28	6	23.39 ± 0.19	23.10 ± 0.21	−0.20 ± 0.29
			$P > 0.10$	$P < 0.01$	$P < 0.10$
			(−10–0)	(40–50)	
No operation	Oct 4	6	23.60 ± 0.29	23.57 ± 0.29	−0.05 ± 0.10
Castration	Oct 4	6	23.21 ± 0.12	23.41 ± 0.12	0.21 ± 0.21
			$P < 0.02$	$P > 0.10$	$P < 0.05$
			(110—120)	(170—180)	
Empty implant	Dec 11	6	23.53 ± 0.25	23.54 ± 0.16	0.00 ± 0.11
Testosterone	Dec 11	5	23.58 ± 0.28	23.36 ± 0.17	−0.22 ± 0.22
			$P > 0.10$	$P > 0.10$	$P < 0.10$
			(180—190)	(240—250)	

Periods are given as means ± SD from 10-day intervals indicated between parentheses; day 0 = May 28; P values are based on two-tailed t-test.

The pathways of this influence are presently unknown. Both castration and testosterone replacement naturally cause major alterations in the animal's endocrine balance. Nothing suggests that it is testosterone itself, instead of, e.g., changes in gonadotropin or gonadotropin releasing factors, which directly affects the central pacemaker. The long time constants of the effects on τ following castration and testosterone implantation (Fig. 3) provide no clue to the mechanism of the effects. Such slow responses of circadian activity rhythms are typical for other agents influencing τ, e.g., the intensity of constant illumination (13).

The change in the distribution of activity within the circadian cycle suggests that our manipulations have caused a disturbance in internal temporal organization. While castration strongly reduces activity during most of the cycle, it leads to an increased peak of activity at the end of the daily activity time. A recent hypothesis on the structure of the pacemaker underlying the circadian activity cycle in higher vertebrates postulates a system of two coupled oscillators or groups of oscillators, one locking on to dawn, the other to dusk (14, 15). These are thought to be responsible for the general bimodality of circadian activity patterns. The opposite effect of castration and testosterone replacement on the amount of activity at the beginning and end of the activity time suggests that the two oscillators are under differential endocrine control. A similar hypothesis has been advanced for birds, where castration and testosterone treatment as well as seasonal testis growth and involution lead to predictable changes in the phase relationship of activity onset and end (starlings; refs. 9 and 16), and annual changes in the relative strength of morning and evening peaks of activity accompany the annual reproductive cycle (finches; ref. 17). Differential endocrine effects on the two oscillating components in the circadian pacemaker, which were postulated to have slightly different natural frequencies, may further account for the small change in τ associated with castration in mice.

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1. Welsh, J. H. (1938) "Diurnal rhythms," Quart. Rev. Biol. **13**, 123–139.
2. Richter, C. P. (1964) "Biological clocks and the endocrine glands," *Excerpta Med. Found. Int. Congr. Ser.* **83**, 119–123.
3. Andrews, R. V. (1971) "Circadian rhythms in adrenal organ cultures," *Gegenbaurs Morphol. Jahrb. Leipzig* **117**, 89–98.
4. Menaker, M. (1974), "Aspects of the physiology of circadian rhythmicity in the vertebrate central nervous system," in *The Neurosciences, IIIrd Study Program*, eds. Schmitt, F. O. & Worden, G. O. (MIT Press, Cambridge, Mass.), pp. 479–489.
5. Takebe, K., Sakahura, M., Horiuchi, Y. & Mashimo, K. (1971) "Persistence of diurnal periodicity of CRF activity in adrenalectomized and hypophysectomized rats," *Endocrinol. Jap.* **18**, 451–455.
6. Rusak, B. & Zucker, I. (1975) "Biological rhythms and animal behavior," *Annu. Rev. Psychol.* **26**, 137–171.
7. Moore, R. Y. & Lenn, N. J. (1972) "A retinohypothalamic projection in the rat," *J. Comp. Neurol.* **146**, 1–14.
8. Pittendrigh, C. S. & Daan, S. (1975) "A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency," *J. Comp. Physiol.*, in press.
9. Gwinner, E. (1974) "Testosterone induces "splitting" of circadian locomotor activity rhythms in birds," *Science* **185**, 72–74.
10. Kincl, F. A., Benagiano, G. & Angee, I. (1968) "Sustained release hormonal preparations 1. Diffusion of various steroids through polymer membranes," *Steroids* **11**, 673–680.
11. Hoskins, R. G. (1925) "Studies on vigor II: The effect of castration on voluntary activity," *Am. J. Physiol.* **72**, 324–330.
12. Richter, C. P. (1927) "Animal behavior and internal drives," *Quart. Rev. Biol.* **2**, 307–343.
13. Daan, S. & Pittendrigh, C. S. (1975) "A functional analysis of circadian pacemakers in nocturnal rodents. III. Heavy water and constant light: homeostasis of frequency?," *J. Comp. Physiol.*, in press.
14. Pittendrigh, C. S. (1974) "Circadian oscillations in cells and the circadian organization of multi-cellular systems," in *The Neurosciences, Third Study Program*, eds. (Schmitt, F. O. & Worden, G. O. (MIT Press, Cambridge Mass.), pp. 437–458.
15. Pittendrigh, C. S. & Daan, S. (1975) "A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker complexity: A clock for all seasons," *J. Comp. Physiol.*, in press.
16. Gwinner, E. (1975) "Effects of season and external testosterone on the freerunning circadian activity rhythms of European starlings," *J. Comp. Physiol.*, in press.
17. Daan, S. (1975) "Light intensity and the timing of daily activity in finches," *The Ibis*, in press.